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Design of Cyclic-ADT Peptides to Improve Drug Delivery to the Brain via Inhibition of E-Cadherin Interactions at the Adherens Junction

By

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Submitted to the graduate degree program in the Department of Pharmaceutical Chemistry and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Sciences.

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Design of Cyclic-ADT Peptides to Improve Drug Delivery to the Brain via Inhibition of E-Cadherin Interactions at the Adherens Junction

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Chairperson: Teruna J. Siahaan

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Abstract

We have developed linear cadherin peptides (i.e., HAV- and ADT peptides) that enhance brain delivery of drug molecules to the central nervous system (CNS). These peptides modulate cadherin interactions in the adherens junctions of the vascular endothelial cells in the blood-brain barrier (BBB) to increase paracellular drug permeation. In this study, the goal was to design cyclic peptides (ADTC1, ADTC5, and ADTC6) derived from linear ADT6 (Ac-ADTPPV-NH_2) to improve their stability and biological activity in improving paracellular delivery of drugs into the brain. The ADTC1 peptide (cyclo(1,8)Ac-CADTPPVC-NH_2) was designed by adding two Cys residues at the N- and C-terminus of ADT6 peptide and a disulfide bond from thiol groups of the Cys residues. The ADTC5 peptide (cyclo(1,7)Ac-CDTPPVC-NH_2) was derived from ADTC1 by deleting the alanine residue from the N-terminal region of ADTC1 and ADTC6. (Cyclo(1,6)Ac-CDTPPC-NH_2) was constructed by deleting the valine residue from the C-terminal region of ADTC5. The results showed that ADTC1 has activity in inhibiting the resealing of the intercellular junctions of the MDCK cell monolayers similar to that of the linear ADT6, indicating that cyclization can maintain the peptide activity. The alanine residue deletion in ADTC5 does not reduce its activity compared to ADTC1 peptide, suggesting that the alanine residue does not have an important role in the activity of the peptide. In contrast, ADTC6 peptide does not have activity in inhibiting the junction resealing, indicating that the valine residue is important for peptide activity. ADTC5 inhibits the junction resealing of MDCK cell monolayers in a concentration-dependent manner with the saturation concentration above 0.4 mM and IC_{50} around 0.3 mM. Under the current experimental conditions, ADTC5 improves the delivery of ^{14}C-mannitol to the brain about two fold compared to the vehicle negative control in the *in situ* rat brain perfusion model. Furthermore, ADTC5 peptide does not enhance the BBB passage of large polyethylene glycol molecules(i.e., PEG-1500 and PEG-40000) in an *in situ* rat brain perfusion model. In conclusion, formation of cyclic peptides can maintain cadherin peptide ability to modulate intercellular junctions of the BBB, and the primary sequence of ADT peptides is important for their biological activity.

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1. INTRODUCTION

Delivering drugs to the brain to treat brain diseases is challenging; this is due to the presence of the blood brain barrier (BBB), which prevents drug transport from the systemic circulation into the brain. Most small and macromolecule drugs can not readily cross the BBB into the brain [1]; this is due to the presence of tight junctions [2], efflux pumps [3, 4], and many metabolism enzymes [5, 6]. Because of their physicochemical properties, peptide and protein drugs cannot readily partition into the cell membranes of endothelial cells and penetrate the BBB passively via the transcellular route [7]. The transcellular pathway of drug transport is also limited by the presence of the efflux pump system, which recognizes a wide variety of drug molecular structures [3]. Finally, the paracellular transport of drug molecules with hydrodynamic diameter larger than 11 angstroms or 500 daltons molecular weight is limited by the presence of tight junctions [8]. Peptide-metabolizing enzymes were more pronounced in the cerebral microvasculature than in whole brain homogenates [5]; therefore, peptide and protein drugs maybe degraded prior to crossing the BBB, preventing the intact drugs from entering the brain. In addition, cytochrome P450 enzymes are expressed at very high levels in the brain astrocytes border, and they metabolize 70–80% of currently used drugs to prevent them from crossing the BBB into the brain parenchyma [6].

Many efforts have been made to cure brain diseases by improving brain delivery of drugs [1, 8, 9]. For example, intracerebro-ventricular injection may provide high drug bioavailability (e.g., close to 100%) in the brain; however, the drug level drops drastically with respect to distance because of cerebrospinal fluid (CSF) diffusivity. Therefore, it requires injections at multiple sites to achieve sufficient drug efficacy [10]**.** Improving the transcellular drug permeation across the BBB has been extensively studied; one of the methods is via cationization

of biopharmaceutical drugs to improve the adsorptive-mediated transcytosis [11]. The presence of insulin, transferrin, and nicotinic acetylcholine receptors in endothelial cells has been exploited to improve brain delivery via drug conjugation to monoclonal antibodies (mAbs) that bind to human insulin and transferrin receptors [1], while conjugation to rabies virus glycoprotein peptide (RVG29) has been used to target the acetylcholine receptor [12]. However, saturation of these receptors during receptor-mediated transcytosis may become a limitation for efficient drug delivery through the BBB. Another method to improve drug delivery through the BBB is enhancing drug permeation through intercellular junctions. Interferring phosphorylation of intercellular junction proteins causes relocation of some proteins at the adherens and tight junctions; this protein relocation generates opening of the paracellular pathways [13]. However, the development of phosphorylation inhibitors as adjuvants to improve paracellular drug permeation is limited by the non-specificity of phosphorylation inhibitors and the complexity of phosphorylation pathways.

Our group investigates cadherin peptides that can inhibit cell-cell adhesion in the adherens junctions of the BBB.HAV peptides derived from the sequence of extracellular-1 (EC-1) domain of E-cadherin have been shown to modulate the paracellular permeation of cell monolayers [14, 15]. An HAV peptide, Ac-SHAVSS-NH_2, has also been shown to improve the paracellular permeation of ^{14}C-mannitol and ^{3}H(G)-daunomycin through the BBB using an *in situ* rat brain perfusion model [16]. ADT-6 peptide was derived from the EC1 domain of E-cadherin as a counter-sequence of the HAV sequence on the EC1 domain. ADT-6 peptide was found to modulate the intercellular junctions of Madin-Darby Canine Kidney (MDCK) cell monolayers and enhance paracellular permeation of ^{14}C-mannitol through cell monolayers. In cell monolayers, the size of ADT peptide can influence its activity because the peptide has to

pass through the tight junctions before it can inhibit cadherin-cadherin interaction at the adherens junctions. ADT hexapeptide was active when added from the apical side of the cell monolayers. In contrast, an ADT decapeptide was not effective in modulating intercellular junctions when it was added from the apical site; this is possibly due its inability to penetrate the tight junctions. Because of the absence of tight junctions on the basolateral side of MDCK cell monolayers, an ADT decapeptide can modulate the adherens junction from that side [15].

In the present work, three cyclic ADT peptides (Table 1) were designed to improve stability and modulatory activity and to evaluate the effects of residue deletion and size of cyclic peptides on their biological activity.

Table 1: Sequence of E-cadherin peptides. Bold C indicates cysteine residues that form a disulfide bond to create a cyclic peptide.

Name	Sequences	MW determined by MS
ADT6	Ac-ADTPPV-NH_2	639.72
ADTC1	Ac-**C**ADTPPV**C**-NH_2	844.00
ADTC5	Ac-**C**DTPPV**C**-NH_2	773.94
ADTC6	Ac-**C**DTPP**C**-NH_2	673.78
KKLVPR	Ac-KKLVPR-NH_2	724.93

To form the ADTC1 cyclic peptide, two cysteine residues were added to the N- and C-termini, respectively, of ADT6; then an intramolecular disulfide bond was generated from the N- to the C-terminus Cys residues. The alanine residue from the N-terminus of ADT6 was deleted to make

ADTC5 for evaluating the effect of the Ala residue on its activity. Finally, the valine residue was deleted from ADTC5 to produce ADTC6 to test the effect of C-terminal valine residue on the activity of peptide. The biological activity of cyclic peptides in modulating the intercellular junctions was evaluated by measuring the change in transepithelial electrical resistance (TEER) values of MDCK cell monolayers in the presence of peptides. The enhancement of paracellular permeation of marker molecules through the BBB of an *in situ* rat brain perfusion model by these cyclic peptides was evaluated.

2. MATERIALS AND METHODS

2.1. Peptide synthesis

Peptides were synthesized using Fmoc chemistry and a Perceptive Peptide synthesizer as previously described [15]. The purification was done by reversed-phase HPLC using a C-18 semi-preparative column. Mass spectrometry was used to determine the correct molecular weight of each peptide. Cyclic peptides were formed via oxidation of the thiol groups of the N- and C-terminus Cysresidues. All of the peptides had a purity of ≥ 95% as confirmed by reversed-phase analytical HPLC using a C-18 column.

2.2. Cell culture

Madin-Darby Canine Kidney (MDCK) cell strain-2 obtained from ATCC (Rockville, MD, USA) was grown in 75 cm^2 Corning polystyrene flasks (Corning, NY, USA). Dulbecco's modification of Eagle's medium (Cellgro, Manasas, VA, USA) enriched with 10% fetal bovine

serum (Atlanta Biologicals, Lawrenceville, VA, USA), 100 units/ml penicilline, and 100 µg/ml streptomycin (Atlanta Biologicals), and 1.42 g/l HEPES sodium was used to culture the cells. The cells were incubated at 37°C, 95% humidity and 5% CO_2. The medium was changed three times a week, and the cells were trypsinized with 0.25% trypsin in 1.0 mM EDTA (Atlanta Biologicals). One-tenth of the harvest cells were seeded in a new polystyrene flask.

2.3. Resealing assay

MDCK cells were seeded at 100.000 cells/well in 0.4 µm polyethylene membrane Transwells (Costar, Cambridge, MA, USA) with diameters of 1.12 cm^2. The medium was changed every other day until the monolayer had TEER values above 300 $Ohm.cm^2$ between days 5and 7. The monolayers were washed three times with medium A (Hank's balances salt solution (HBSS), pH 7.4, enriched with 10 mM HEPES, 1% glucose, and 2.0 mM$CaCl_2$). The last washing solution was kept in the wells for 1.5 hours to equilibrate the cells before TEER measurements. TEER values were measured at the end of the equilibration time, which was defined as t = 0 hour. To open the intercellular junctions, equilibrated monolayers were washed three times with medium B (the same as medium A but without $CaCl_2$) and incubated for 1.0 hours using the last washing solution. At the end of the incubation, TEER values were measured using EVOM2 (World Precision Instrument, Sarasota, FL) as t = 1 hour. Following the junction opening, the apical and basolateral chambers were incubated in 1.0 mM peptide dissolved in buffer A. The change in TEER value was measured every hour up to 6 hours. Buffer A and non-cadherin peptide (i.e., KKVPR) were used as a negative control. The experiment was performed at least in triplicate.

2.4. *In situ* rat brain perfusion

Rats (55–60 days old) were kept in The Animal Care Unit, The University of Kansas, under free access to food and water. The *in situ* rat brain perfusion was performed according to a Takasato publication with some modifications [17]. Briefly, rats were anesthetized intraperitoneally with 100 mg/kg of ketamine and 5 mg/kg of xylazine. Polyethylene catheters (PE 50) containing heparin-saline (10 IU/ml) were used to cannulate the left common carotid artery (LCCA), and retrograde perfusion was performed to prevent coagulation. During cannulation of the LCCA, the left pteryopalatine, occipital, and superior thyroid arteries were ligated. A heat lamp was used to maintain therat's body temperature. Before perfusion, rats were decapitated with cardiac puncture. The LCAA was washed for 10 sec using heparin-saline solution followed by 10ml perfusion of 1.0 mM peptide dissolved in HCO_{3-}buffered physiological saline containing 0.5% v/v Tween 20. The HCO_{3-}buffered physiological saline was composed of 142 µmol/ml NaCl, 28.0 µmol/ml $NaHCO_3$, 4.2 µmol/ml KH_2PO_4, 1.7 µmol/ml $CaSO_4$, and 1.0 µmol/ml $MgSO_4$ and was aerated with 5% CO_2 at 37ºC temperature and 95% humidity prior to the experiment. Following the peptide perfusion, 10 µCi^{14}C-mannitol (Moravek Biochemicals, Brea, CA) dissolved in heparin-saline solution containing 0.5% v/v Tween 20 was delivered. At the end of perfusion, a 10-sec post-perfusion wash using saline-heparin solution was performed to remove the excess ^{14}C-mannitol in the brain microvessels. All perfusions were done at 5 ml/sec rate using an infusion pump (Model 355 syringe pump, Sage Instrument, Boston, MA). Rat brain was removed from the skull and divided into three parts. Each part of the brain was weighed and ground. Four milliliters of scintillation fluid (Fisher Scientific, Morris Plains, NJ, USA) was added to each sample. The radioactive activity was measured using a dual-double scintillation spectrometer (Beckman LS 6000 IC).

Transport studies with polyethylene glycol (PEG) molecules were performed using the same techniques as described above. 1,2-^{3}H-PEG-1500 and 1,2-^{14}C-PEG-40000 (American Radioactive Chemicals, St. Louis, MO) with strength 10 µCi/rat were used to evaluate the limits of pore-size openings created by cadherin peptides in *in situ* rat brain perfusion experiments.

3. RESULTS

3.1. Effect of cyclic peptide formation and residue deletion on biological activity

The biological activities of a linear peptide (i.e., ADT6) and cyclic peptides (i.e., ADTC5 and ADTC6) were compared to those of a negative control (HBSS medium) in the junction-resealing assay using MDCK cell monolayers (Figure 1). In this assay, intercellular junctions were opened upon temporarily removing calcium ions from the medium. Under this condition, calcium-dependent junctional proteins are unable to form homotypic interactions in the adherens junctions; as a result, the TEER values drop upon opening of the intercellular junctions. After 1 hour, the junctions can be resealed upon addition of calcium ions into the medium. During the resealing, the peptide was added to determine its ability to block calcium-dependent cadherin-cadherin interactions. When the peptide blocks the cadherin-cadherin interactions, the resealing of the intercellular junctions is inhibited; as a result, the TEER values of the monolayers do not return to their original values as they do in HBSS (negative control).

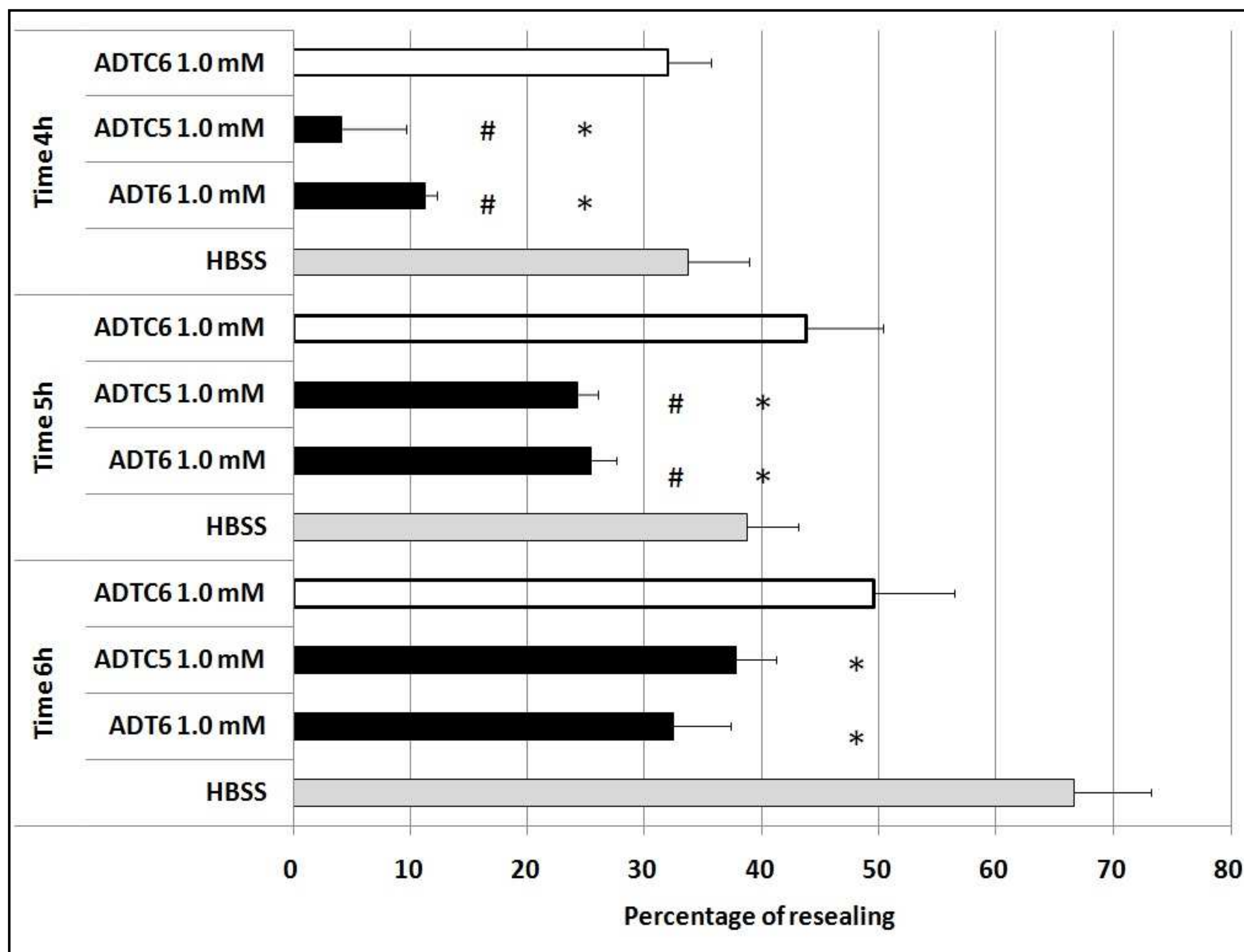

Figure 1: Effect of incubation time on percent change of TEER values during resealing inhibited by 1.0 mM of ADT6, ADTC5, and ADTC6 at different time points (4h to 6h). Linear ADT6 produced significantly lower resealing compared to HBSS at 4h, 5h and 6h (p<0.05) time points. ADTC5 caused a significantly lower percent of resealing compared to HBSS at time points 4h, 5h, and 6h (p<0.05). ADTC6 did not have any difference in percent of resealing compared to HBSS at any time point. Significant differences were observed between ADT6 and ADTC6 and between ADTC5 and ADTC6 at a concentration of 1.0 mM at 4h and 5 h time points (p <0.02). Generally, there was no significant difference between linear ADT6 and ADTC5 at 1.0 mM concentration. The experiments were done in triplicate, and the values are the mean ± SE. Star (*) means significantly different from HBSS; number sign (#) means significantly different from 1.0 mM ADTC6.

At 1.0 mM concentration, linear ADT6 and cyclic ADTC5 have comparable inhibitory activity in inhibiting junction resealing from the 4h to the 6h time point ($p<0.05$). This suggests that cyclization does not increase or abolish the activity of ADTC5.The activity of 1.0 mM ADT6 and ADTC5 is significantly different from that of 1.0 mM ADTC6 ($p<0.01$). In contrast, there is no significant difference between ADTC6 and HBSS, suggesting that ADTC6 does not have the ability to inhibit resealing of the intercellular junctions (Figure 1). This signifies the importance of some residues in the peptide. The inactivity of 1.0 mM ADTC6 was also seen in another experiment when it was compared to a negative control KKLVPR, which is derived from another cell adhesion protein called intercellular adhesion molecule-1 (ICAM-1) (data not shown).

The activity of ADTC5 peptide at two different concentrations (0.5 and 1.0 mM) was compared to that of a control peptide (KKLVPR) (Figure 2). A trend showing that KKLVPR causes a lower percentage of resealing compared to HBSS was observed; however, there was no statistically significant difference between HBSS and KKLVPR treatments ($p>0.05$) at the 6h time point. The activity of 1.0 mM ADTC5 was significantly different from that of both 1.0 mM KKLVPR ($p<0.05$) and HBSS ($p < 0.01$), confirming that ADTC5 has specificity for E-cadherin in the intercellular junctions. Although there was a trend to show that the inhibitory activity of 1.0 mM ADTC5 is better than that of 0.5 mM ADTC5, there was no statistically significant difference between their activities at the 6h time point. This finding suggested that 0.5 mM of ADTC5 might have saturated the site of binding of the protein receptors (i.e., E-cadherin); this proposal was confirmed later during concentration-dependent study.

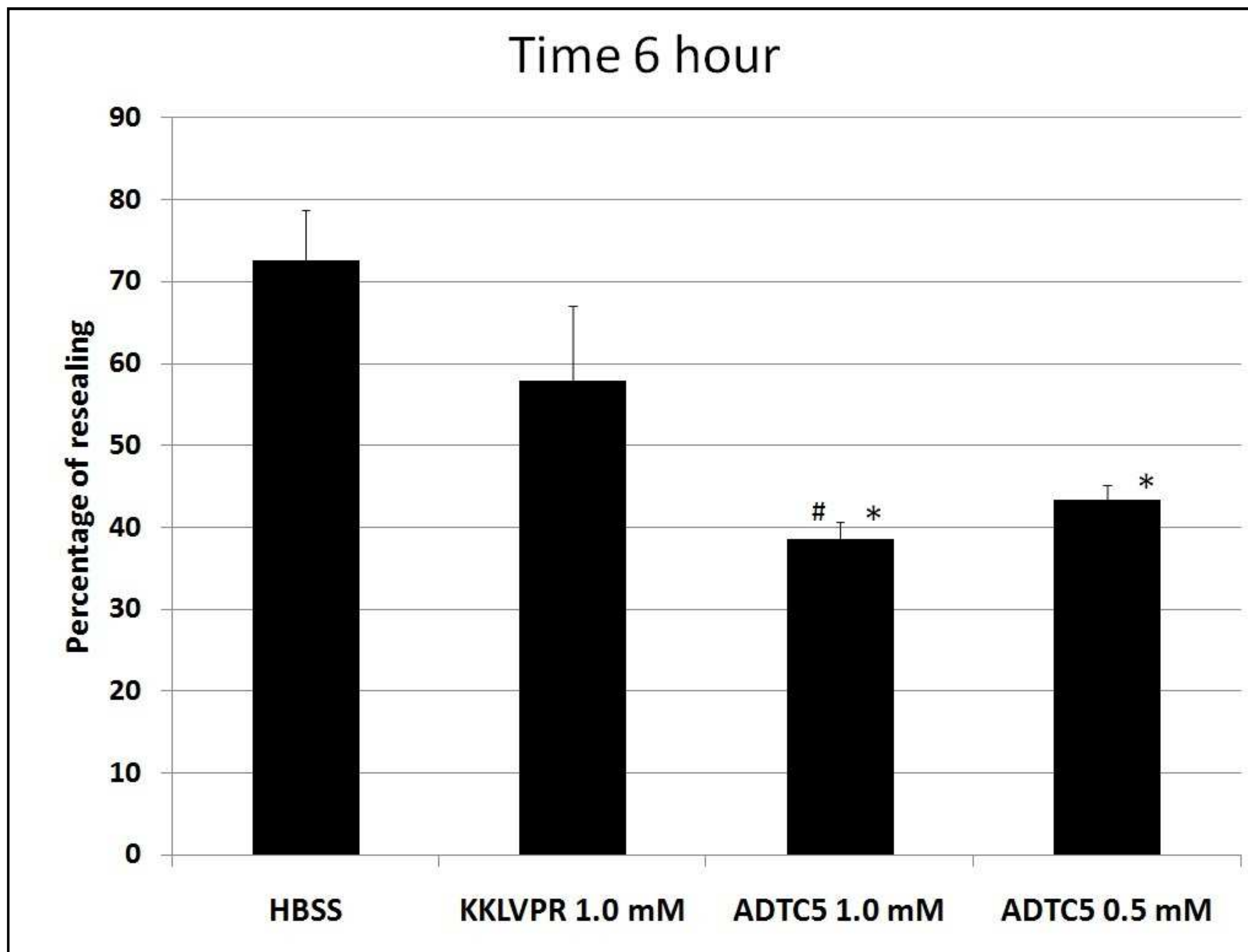

Figure 2: Inhibitory activity for junction resealing of ADTC5 (0.5 and 1.0mM) compared to 1.0 mM control peptide (KKLVPR) and HBSS in MDCK monolayers. At the 6h time point, the ADTC5 peptide (at 0.5 and 1.0 mM) blocks the resealing of MDCK cell monolayers significantly better than 1.0 mM KKLVPR peptide and HBSS. There is no significant difference between KKLVPR and HBSS ($p>0.05$). No significant difference in activity was observed between 1.0 mM and 0.5 mM of ADTC5 at any time points ($p>0.05$). A star symbol (*) means significantly different from HBSS; a number sign (#) means significantly different from 1.0 mM KKLVPR.

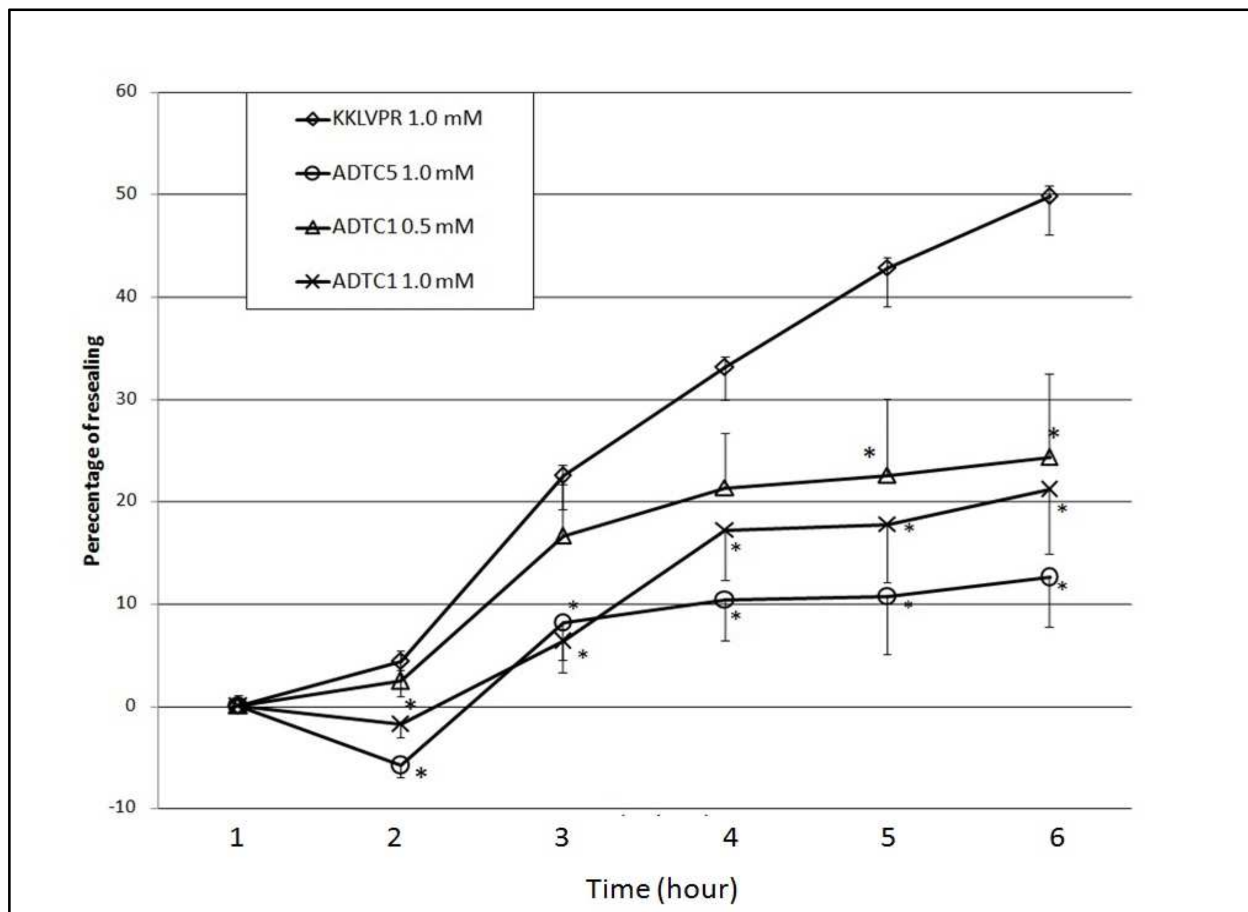

Figure 3: Comparison of the biological activity of ADTC1 and ADTC5 to that of a negative control (KKLVPR). The biological activities of 0.5 mM ADTC1 (Δ), 1.0 mM ADTC1 (×) and 1.0 mM ADTC5 (○) were significantly different from that of KKLVPR (◊). There is no significant difference between 1.0 mM ADTC5 and1.0 mM ADTC1 at any time points. There is no significant difference between the activity of 0.5 mM ADTC1 and 1.0 mM ADTC1 from 3–6 h time points (p>0.05). A star symbol (*) means a significant difference from 1.0 mM KKLVPR.

The activities of the cyclic peptides were compared to find the best peptide for *in situ* rat brain perfusion studies (Figure 3). First, the activity of ADTC1 at two different concentrations (0.5 and 1.0 mM) was compared to that of 1.0 mM ADTC5 and 1.0 mM KKLPVR (a negative control). ADTC1 effectively inhibits the junction resealing compared to the negative control. There was a trend indicating that ADTC5 may be a better inhibitor than ADTC1 ($p<0.34$) and that the activity of ADTC1 is concentration-dependent ($p<0.14$).

3.2. IC_{50} determination of ADTC5

Because ADTC5 is the best peptide thus far, the concentration-dependent activity was investigated to determine its IC_{50} compared to that of a negative control peptide (KKLVPR). In this case, different concentrations of ADTC5 and KKLVPR were added into cell monolayers and the TEER values were measured up to the 6 h time point. The TEER value of peptide was compared to that of HBSS to determine the percent of resealing, which is assumed to be 100%. The results showed that inhibition by ADTC5 was saturated at concentrations above 0.4 mM with resealing only up to 26%, and the IC_{50} of ADTC5 was observed at a concentration around 0.3 mM. In contrast, KKLVPR had no significant activity in inhibiting the resealing compared to the HBSS medium at any concentration. Previous experiments (Figure 2) also confirmed that up to 1.0 mM KKLVPR did not have activity significantly different from that of HBSS medium (p >0.05).

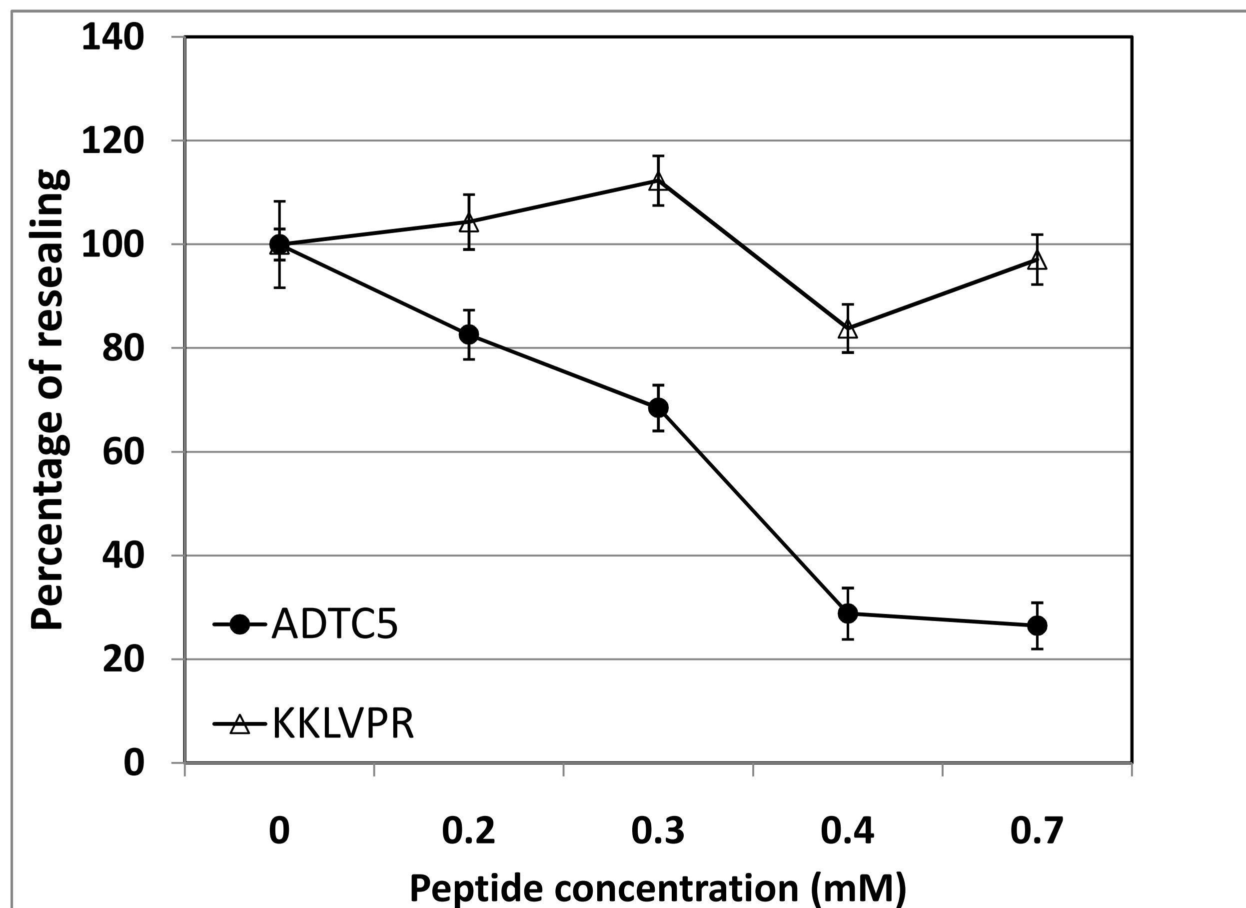

Figure 4: The concentration-dependent activity of ADTC5 (●) and KKLVPR (∆) to inhibit the junction resealing of MDCK cell monolayers. Each data point represents the relative TEER values between the peptide-treated and medium-treated cell monolayers after 6 hours intercellular junction resealing. The value is mean ± SE of six replicates. The inhibition of MDCK resealing by ADTC5 was saturated at a concentration of 0.4 mM, and KKLVPR did not affect the resealing up to a concentration of 0.7 mM.

3.3. The activity of ADTC5 to enhance the delivery of paracellular marker molecules to the brain in *in situ* rat brain perfusion model

The ability of ADTC5 to open the BBB was evaluated in enhancing ^{14}C-mannitol transport to the brain in an *in situ* rat brain perfusion model. ADTC5 at concentration 1.0 mM can enhance the transport of mannitol 1.93-fold compared to vehicle control (p<0.01) as shown in Figure 5. As a positive control, linear SHAVAS with the same concentration as ADTC5 was able to improve the delivery of mannitol about 1.70-fold compared to control vehicle (p<0.02). There was a trend showing that cyclic ADTC5 may have better modulatory activity than linear SHAVAS (p<0.40); however, further studies need to be carried out to evaluate the difference between these two peptides.

To evaluate the limit of pore size opening created by ADTC5, the BBB permeation of large paracellular marker molecules (i.e., ^{3}H-PEG-1500 and ^{14}C-PEG-40000) in *in situ* rat brain perfusion was determined in the presence and absence of ADTC5 peptide. There was no significant difference between the transport of PEG-1500 in the presence and absence of ADTC5 peptide (Figure 6); the K_{in} values of PEG-1500 with or without peptide were 0.34×10^{-3} and 0.42×10^{-3} ml.min^{-1}.gram^{-1}, respectively. Similarly, there was no significant difference in the transport of PEG-40000 in the presence and absence of ADTC5 peptide (Figure 6); the K_{in} values of PEG-40000 with or without peptide were 0.07×10^{-3} and 0.08×10^{-3} ml.min^{-1}.gram^{-1}, respectively. As expected due to their respective size, the K_{in} of PEG-1500 is higher than that of PEG-40000. As a perspective, the K_{in} values in this study using rat as a model are slightly higher than those of PEG-4000 in guinea pig, which is 0.004×10^{-3} ml.min^{-1}.gram^{-1} [18].

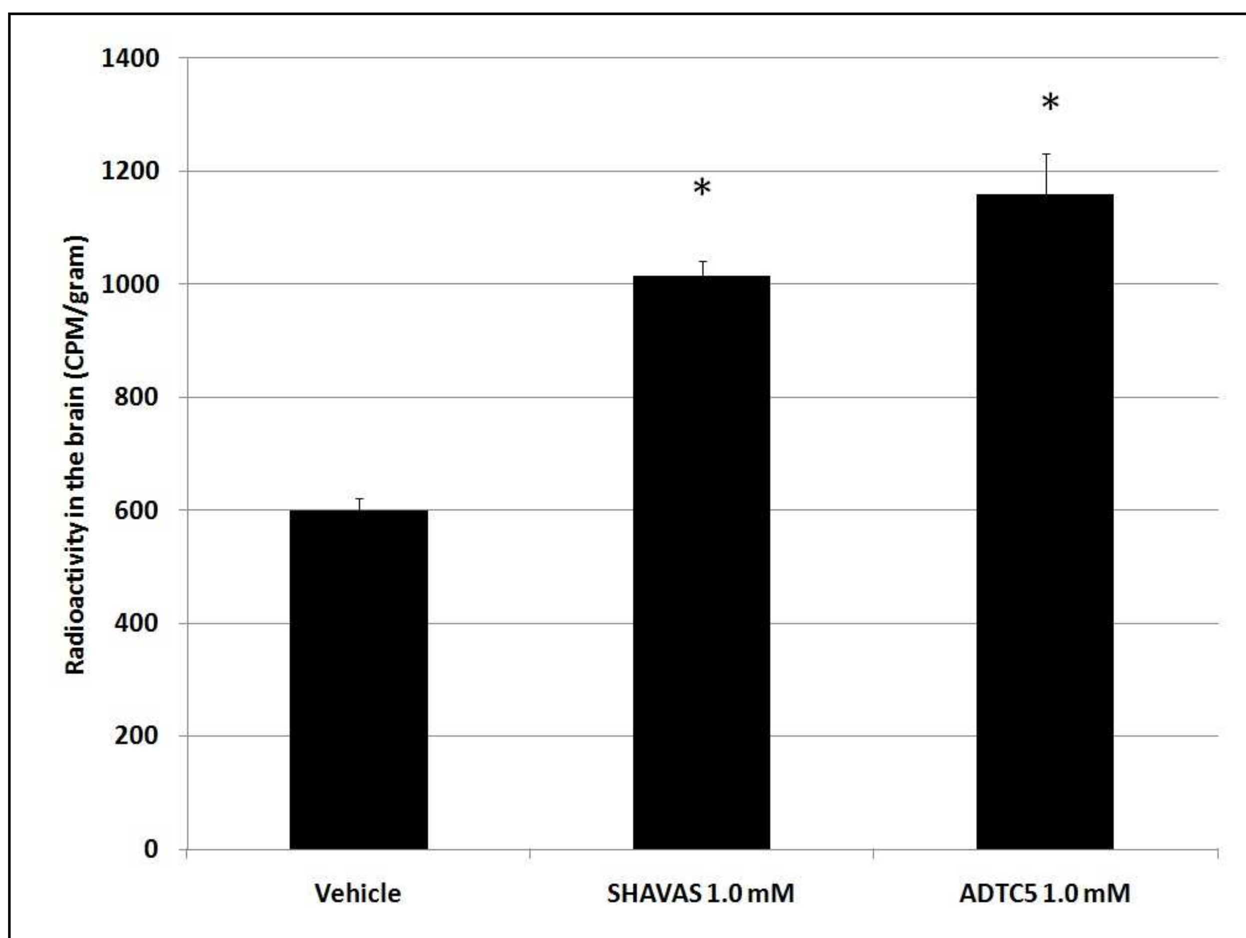

Figure 5: Level of radioactive ^{14}C-mannitol in the brain after *in situ* rat brain perfusion. The brain was perfused with 1.0 mM of peptide prior to delivery of 10 ml of^{14}C-mannitol1 µCi/ml at a rate of 5 ml/min. The value was the mean ± SE of three replications. The star indicates a significant difference from the vehicle.

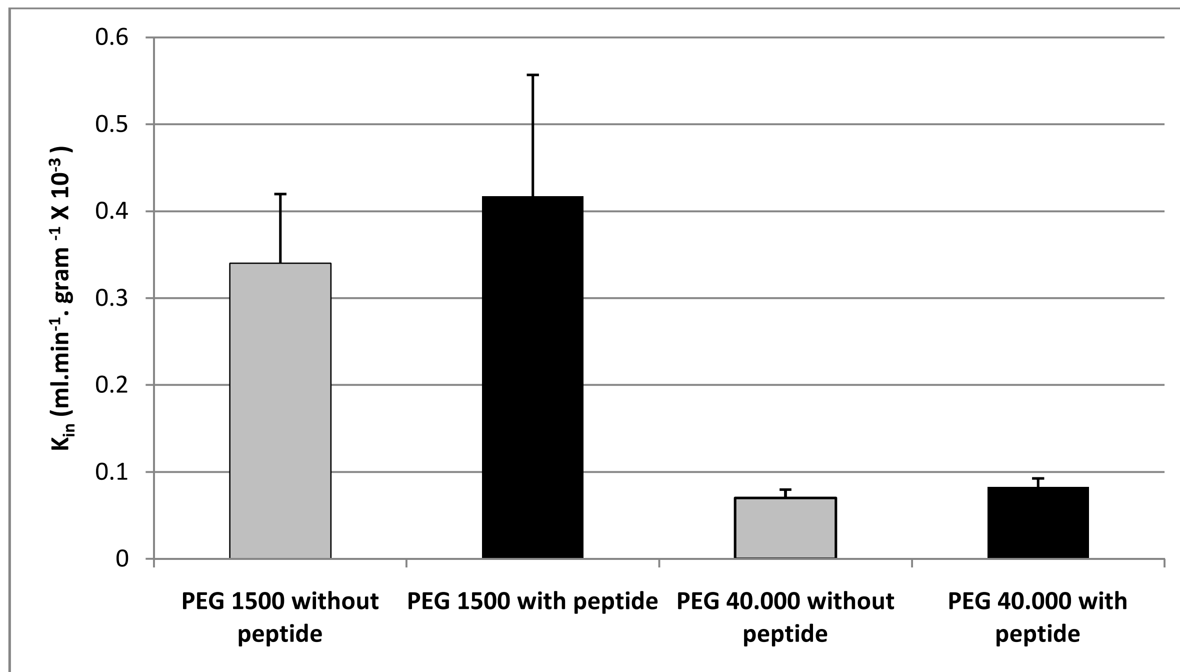

Figure 6: K_{in} of ^{3}H-PEG-1500 and ^{14}C-PEG-40000 calculated after perfusion with or without perfusion of ADTC5 peptide. ADTC5 1.0 mM was perfused at a rate of 5 ml/min for 2 min followed by a 2 minute perfusion of 1.0 µCi/ml radioactive PEG at the same rate. The experiment was done in triplicate. K_{in} value was the mean ± SE. There was no significant improvement in PEG-1500 and PEG-40000 transport to the brain with or without peptide (p >0.05).

4. DISCUSSION

In the last decade, linear ADT6 peptide derived from the counter sequence of HAV peptide on the EC1 domain of E-cadherin was found to modulate the intercellular junctions of colorectal adenocarcinoma-2 (Caco-2) and MDCK cell monolayers. ADT peptide increases the porosity of intercellular junctions, which is reflected by the decrease in TEER values and improvement of apparent permeability (P_{app}) of ^{14}C-mannitol through the MDCK monolayers [15]. Therefore, this study was designed to improve the biological activity as well as the chemical and biological stability of ADT peptides. Several ways to improve the activity and metabolic stability include formation of cyclic ADT peptides and creating derivatives of cyclic ADT peptide via residue mutation and/or deletion. In this study, three cyclic peptides (i.e., ADTC1, ADTC5, and ADTC6) were synthesized and evaluated for their ability to inhibit junction resealing of MDCK monolayers. The ability of the best cyclic peptide (i.e., ADTC5) to improve transport of paracellular markers (i.e., mannitol, PEG-1500, and PEG-40000) was determined.

In the resealing assay of MDCK monolayers, conversion of linear ADT6 (ADTPPV) to cyclic peptides (i.e., ADTC1 and ADTC5) maintains its bioactivity in inhibiting the junction resealing. Previous studies have shown that formation of cyclic peptides could enhance or maintain their biological activity and/or chemical stability [19]; however, formation of cyclic peptides could result in a loss of activity due to formation of an unfavorable conformation for binding to the receptor [20]. In this study, deletion of the alanine residue from ADTC1 to make ADTC5 (CDTPPVC) did not affect the biological activity (Figure 3), suggesting that the alanine residue is not important for peptide activity or its binding to E-cadherin. Another possible explanation is that the side chain of the N-terminus cysteine residue could act as a substitute for

the side chain of the alanine residue that binds to the hydrophobic pocket of EC-domain of E-cadherin. ADTC5 peptide inhibits junction resealing in a concentration-dependent manner with saturation around 0.4 mM concentration; the IC_{50} of inhibition is around 0.3 mM (Figure 4). Furthermore, deletion of C-terminus valine residue in ADTC5 generates cyclic ADTC6 peptide (CDTPPC); unfortunately, ADTC6 peptide lost its biological activity completely, indicating that the valine residue is an important residue for the biological activity and for its binding to E-cadherin.

ADTC5 peptide at concentration 1.0 mM significantly enhances BBB permeation of ^{14}C-mannitol (1.93-fold) in an *in situ* rat brain perfusion model compared to vehicle; it has activity to enhance transport similar to that of a positive control, HAV peptide (Ac-SHAVAS-NH_2) 1.0 mM, which produces 1.70-fold transport enhancement compared to vehicle control. To evaluate the pore-size limit of the intercellular junction opening mediated by ADTC5 peptide, the transport of PEG-1500 and PEG-40000 was evaluated in the absence and presence of peptide in *in situ* rat brain perfusion model. A very large opening of the intercellular junctions of the BBB by cadherin peptides may create a problem of diffusion of unwanted large proteins, particles, and immune cells into the central nervous system (CNS) to create brain toxicity. There was no significant enhancement of BBB transport PEG-1500 and PEG-40000 ($p>0.05$) in the presence of ADTC5 compared to a vehicle negative control, indicating that, using the current experimental conditions, the pore opening caused by ADTC5 does not allow molecules the size of PEG-1500 or larger to pass through the BBB. It should be noted that the application of ADTC5 for two minutes may be too short a time to create large size pore openings that would allow enhancement of permeation of PEG-1500 through the BBB. There is a possibility that lengthening the peptide incubation time may increase the paracellular pore size opening; this is

currently under investigation. Due to the hydrogen-bonding potential of PEG molecules, the hydrodynamic radius of PEG-1500 or PEG-40000 is larger than the size of the molecule itself; for example, PEG-40000 has a hydrodynamic radius 14 times bigger larger than the actual radius of the molecule alone [21]. Therefore, other types of molecules with different sizes and shapes should also be used to estimate the limits of pore size openings in the intercellular junctions of the BBB mediated by cyclic ADTC5 peptide.

As shown previously, opening of paracellular pathways may also be useful in enhancing BBB permeation of hydrophobic drugs that are subjected to the P-glycoprotein (PgP) efflux pump. Modulation of the intercellular junctions with HAV peptide has an effect similar to blocking the efflux pumps by delivering daunomycin into the brain in the *in situ* rat perfusion model. Saturating PgP with verapamil in combination with opening of paracellular pathways by the HAV peptide can improve brain delivery of daunomycin up to five fold [16]. In the future, ADTC5 will also be investigated for enhancing BBB permeation of drugs that are recognized by efflux pumps. Recently, opening of the BBB by cadherin peptides to enhance BBB molecule permeation was also confirmed by magnetic resonance imaging (MRI) experiments. In this case, MRI detects the amount of gadolinium-DTPA (Gd-DTPA) complex that enters the brain upon peptide treatment in an *in situ* rat brain perfusion model. The result showed that there was a significant enhancement of Gd-DTPA in the brain upon peptide treatment compared to control vehicle; this result confirms the bioactivity of cadherin peptides in enhancing ^{14}C-mannitol transport into the brain.

The proposed mechanism of action of cadherin peptides is that they bind to the extracellular (EC) domain of E-cadherin to block cadherin-cadherin interactions. From NMR and X-ray structural studies, each EC repeat domain (EC1 through EC5) has been shown to have

similar beta-barrel structure with a high level of sequence homology [22]. Each EC domain contains a bulge and a groove region, and we propose that cadherin peptide can bind to either region of each EC domain. Molecular modeling studies suggest that ADT peptides bind to the groove region of the EC1 domain of E-cadherin where the HAV sequence resides [15]; it is hypothesized that the side chain of valine in ADT peptide is anchored to the hydrophobic pocket of the groove region of the EC1 domain of E-cadherin. Recently, our NMR studies indicated that ADTC5 and ADTC1 bind to the expressed ^{15}N-labeled EC1 domain of E-cadherin. Both of these peptides caused changes in chemical shifts of ^{1}H and ^{15}N of several residues within the EC1 domain. NMR studies also showed that HAV peptide (Ac-SHAVSS-NH_2) binds to the EC1 domain of E-cadherin at a different site than do ADT peptides; this conclusion was drawn because resonances from different residues were shifting when EC1 was titrated with the HAV peptide compared to when titrated with ADT peptides. It is possible that the mechanism of action of ADT and HAV peptides is due not only to binding to EC1 but also to simultaneous binding to different EC domains (EC1, EC2, EC3, EC5, and EC5) within E-cadherin. Previously, NMR and FTIR studies showed that Ac-SHAVSS-NH_2 and Ac-TYRIWRDTAN-NH_2 (BLG4 from the bulge region of EC4) peptides bind to the EC5 domain of E-cadherin, suggesting that the mechanism of action of cadherin peptide is not only via binding to one repeat domain of E-cadherin [23]. The NMR and molecular docking data also suggest that these two peptides bind to different region of EC5; this is consistent with the proposal that a peptide from the groove region such as Ac-SHAVSS-NH_2 can bind to the bulge region of the EC domains (i.e., EC1 and EC5). In contrast, peptides from the bulge region such as BLG4 can bind to the groove region of the EC domain (i.e., EC1 and EC5). In the future, we will compare the binding characteristics of HAV and ADT peptides to other EC domains (EC2, EC3, and EC4).

In cell culture, binding of E-cadherin peptide causes changes in gene expression of E-cadherin protein expressionin Caco-2 cell monolayers. Although the expression was not altered up to 8 hours after exposure, E-cadherin expression declined 24 hours after peptide exposure and the expression came back up to 90% after 48 hours [24]. The decline of E-cadherin expression was found to be similar upon peptide treatment of MDCK cells [14]. The inhibition of homotypic interaction could be correlated with the internalization of E-cadherin, which promotes E-cadherin degradation in cytosol. The disappearance of E-cadherin from the cell surface was correlated with the gene expression of F-box and leucine-rich repeat protein 4, which encodes the component of E3 ubiquitin ligase that has a role in E cadherin endocytosis [24]. Although tight junctions are above the adherens junction where the E-cadherin resides, the effect of peptide in the expression and internalization of E-cadherin could modulate the interaction of tight junction proteins (i.e., claudins and occludins) to increase paracellular porosities. It has been shown that adherens junctions are connected to tight junctions via tight junction proteins ZO-1 and α-catenin [25]; there is possible crosstalk between adherens junctions and tight junctions through connections of cytoskeletal proteins.

Because the vascular endothelial cells of the BBB contain N- and VE-cadherin, it is still a question whether cadherin peptides from E-cadherin could also bind and modulate VE cadherin interactions. It was confirmed that VE-cadherin is responsible for homotypic interactions while N-cadherin's role might be that of anchoring vascular endothelial cells to its surrounding cells [26]. Our previous studies indicated that fluorescence-labeled HAV peptide and anti-E-cadherin antibody bind to and decorate the intercellular junctions of bovine-brain microvessel cell (BBMEC) monolayers, suggesting the presence of E-cadherin. Another possibility is that the anti-E-cadherin antibody cross-reacts with VE-cadherin in BBMEC as was seen in the BBMEC

aggregation studies [27]. The question is why E-cadherin peptides were able to improve mannitol transport in *in situ* rat brain perfusion even though it was suggested that only N- and VE-cadherin are available in the endothelial cells of the BBB. Although ADT sequences were not available in N- and VE-cadherin, the HAV sequence does exist in E- and N-cadherin peptides (AHAVSS and AHAVDI, respectively) [28, 29] while VE-cadherin has AVIVDK for its sequence homology. The presence of HAV sequence homology in the groove regions of N-cadherin and VE-cadherin suggests that ADT peptide may still bind to VE- or N-cadherin to modulate the BBB. We are currently expressing the EC1 domain of VE-cadherin to evaluate whether HAV and ADT peptides can bind to the EC1 domain of VE-cadherin. These binding studies will be carried out using NMR and other spectroscopic methods (i.e., fluorescence and FTIR). Furthermore, the binding affinities of HAV and ADT peptides to the EC1 domain of E- and VE-cadherin will be compared.

Other peptides have also been explored for improving the paracellular permeation of molecules across the BBB. We found that BLG4peptide from the EC4 of E-cadherin could also modulate cadherin-cadherin interactions [16]. H-SWELYYPLRANL-NH_2peptide was reported to disrupt the integrity of cell monolayers and inhibit the aggregation of cells that expressed only E-cadherin (MCF-7 cell) and N-cadherin (MDA-MB35 cell) [30]. In addition to cadherin peptides, some peptides were also designed to modulate the interaction of other junctional proteins. Cell adhesion recognition (CAR) sequence of occluding was identified as LYHY; linear and cyclic LYHY peptides have been shown to inhibit the adhesion of fibroblast cells transfected with occludin [31]. A twenty-seven amino acid peptide derived from the first loop of claudin-1 can improve the *in vitro* cell permeation of 3 KDa FTIC-dextran. However, its effect on mannitol

and lactulose gastrointestinal transport *in vivo* was less dramatic; this is probably due to the rapid metabolism of the peptide *in vivo*[32].

The alteration of cytoskeleton rearrangement can also be used to disrupt the BBB. Instead of inhibiting junctional protein-protein interactions, another effort to modulate the biological barriers is by relocating the intercellular junction proteins. Protein kinase-Cζ (PKCζ) is responsible for protein relocation via binding directly to occludin and phosphorylation of occludin at the following residues: Thr438, Thr403, Thr404, and Thr424. Application of PKCζ pseudosubstrate (Myr-SIYRRGARRWRKL) reduces the state of occludin phosphorylation and affects occludin redistribution in the tight junctions[33]. This disrupts the tight junction integrity as observed by the lowering of TEER values and increased inulin transport across MDCK monolayers. An FDA-approved adenosine agonist (Lexiscan) can alter RhoA and Rac1 and cause cellular cytoskeleton rearrangement accompanied by reduced expression of occludin, claudin-5, and ZO-1 from cell surfaces [34]. AT1002 peptide was also found to modulate the intercellular junctions of Caco-2 and the BBB cell monolayers; this peptide redistributes ZO-1 from the tight junctions upon tyrosine phosphorylation of ZO-1 via Src kinase activation. Removal of AT1002 was accompanied by recovery of unphosphorylated ZO-1 to normal levels [35] .These approaches have not yet been successful in the clinic in improving drug delivery through the BBB; this is due to many factors, including (a) lack of peptide stability *in vivo*, (b) lack of modulatory selectivity, and (c) limited understanding of their mechanisms of action.

5. CONCLUSIONS

We have discovered cyclic ADT peptides that retain their activity compared to the parent linear ADT6 in modulating intercellular junctions in *in vitro* and *in situ* rat brain perfusion models. This study also showed that the C-terminus valine residue is an important residue for the biological activity of ADT peptide. The opening of the BBB by ADTC5 is limited to molecules with molecular weight less than 1500 daltons. In the future, we will study the activity of ADTC5 peptide to improve brain delivery of drugs recognized by PgP efflux pumps. The binding site of cyclic ADT peptides to EC1 of E-cadherin will also be determined using 3D NMR and molecular modeling studies. Finally, binding of ADT peptides to VE-cadherin will be also be confirmed by NMR studies.

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